

## CHAPTER 5

# **Mechanism of the Electrocatalytic Reduction of Ferricyanide by Methylene Blue at DNA-Modified Electrode Surfaces**

This work was completed in collaboration with Mike Hill in the Chemistry Department at Occidental College. Manuscript in preparation.

## INTRODUCTION

We have developed electrochemical assays of DNA-mediated charge transport at DNA films (1-7). This thesis work has focused on developing this assay as a platform for the development of applications for DNA based sensing. Using electrocatalysis at DNA films, we have developed an extremely sensitive assay for the detection of mismatches and other base stacking perturbations (Chapters 2 and 3, references 5 and 6). In these experiments, DNA oligonucleotide duplexes are modified with a thiol linker and self-assembled on gold electrode surfaces. The redox active intercalator methylene blue ( $\text{MB}^+$ ) is used as the electrochemical probe.  $\text{MB}^+$  reversibly binds to DNA-modified surfaces with an association constant of  $3.8(5) \times 10^6 \text{ M}^{-1}$  (1) and is reversibly reduced at  $-250 \text{ mV vs. SCE}$  with linear peak current vs. scan rate dependence, an indication that  $\text{MB}^+$  is adsorbed to the DNA film. Reduced  $\text{MB}^+$  serves as a catalyst for the reduction of ferricyanide ( $\text{Fe}(\text{CN})_6^{3-}$ ), a negatively charged species diffusing in solution outside of the DNA film. Once reoxidized by ferricyanide, methylene blue is again available for subsequent electrochemical reduction and the catalytic cycle continues. The electrochemical reduction of  $\text{MB}^+$  takes place via charge transport through the DNA base pair stack (Chapter 4). It is important that  $\text{MB}^+$  is a DNA intercalator, as intercalators bind to DNA by slipping in between the DNA base pairs, effectively becoming members of this  $\pi$ -stack (Chapters 2 and 4). We consider that electrocatalysis is particularly sensitive to small

perturbations in base stacking, because during an electrocatalytic assay, the  $\pi$ -stack is repeatedly interrogated each time  $\text{MB}^+$  is reduced electrochemically.

Variation of the catalyst used to reduce  $\text{Fe}(\text{CN})_6^{3-}$  (Chapter 2) as well as  $\text{MB}^+$  concentration (Chapters 2 and 3) and scan rate dependence studies (Chapter 2) have yielded some information concerning the mechanism of the  $\text{MB}^+/\text{Fe}(\text{CN})_6^{3-}$  electrocatalytic reaction. These studies are consistent with a mechanism in which reduced  $\text{MB}^+$  leaves the DNA film to reduce  $\text{Fe}(\text{CN})_6^{3-}$  in solution and then diffuses back into the film to continue in the catalytic reaction. Rotated disk electrochemistry has previously been used to study the mechanism of reactions in which an electrochemically reduced molecule that is bound to an electrode surface catalytically reduces a molecule in solution (8). Here we apply this technique to investigate the mechanism of the catalytic reduction of ferricyanide by methylene blue at DNA-modified surfaces in greater detail.

## MATERIALS AND METHODS

### *Materials*

Unless otherwise indicated, all reagents and solvents were purchased in their highest available purity and used without further purification. All DNA synthesis reagents were obtained from Glen Research.

### *Preparation of DNA-modified surfaces*

Thiol-derivatized DNA duplexes were prepared as described in the Appendix. Briefly, oligonucleotides immobilized on a controlled pore glass resin were treated in succession with carbonyldiimidazole and 1,6-diaminohexane at the 5'-OH terminus before cleavage from the resin. After deprotection, the free amine was treated with 2-pyridyldithiopropionic acid N-succinimide ester to produce a disulfide. Sequences were purified by reversed phase HPLC, converted to free thiols using dithiothreitol, and repurified before hybridization to their complements. The thiol-modified sequence used in this study is 5'-SH-AGTACAGTCATCGCG-3'. When hybridized to its perfect complement, the duplex is referred to as TA, hybridized to a complement with a CA mismatch at the boldface C is referred to as CAtop, and hybridized to a complement with a CA mismatch at the underlined C is referred to as CAbottom.

Solutions containing 0.1 mM duplexes in 5 mM phosphate buffer (pH 7), 50 mM NaCl, and 0.1 M MgCl<sub>2</sub> were deposited on commercial gold disk electrodes (Pine Instruments) that had been polished using 0.05 mM alumina, sonicated, and electrochemically etched in 0.1 M H<sub>2</sub>SO<sub>4</sub> by cycling between 1.4 and -0.4 V vs. SCE. Self-assembly was allowed to occur for 12-24 hours at room temperature in a humidified chamber.

### *Electrochemical measurements*

All electrochemical experiments were performed using either a Bioanalytical Systems (BAS) Model CV50-W electrochemical analyzer or a Princeton Applied Research (PAR) Model 173 potentiostat/galvanostat

controlled by a Model 175 universal programmer. Cyclic voltammetry (CV), linear sweep voltammetry, and chronocoulometry (CC) were performed at ambient temperature with a normal three electrode configuration consisting of a gold disk working electrode (Pine Instruments), an SCE reference electrode, and a platinum gauze auxiliary electrode. Rotated disk data were collected using a Pine Instruments Model AFMSRX analytical rotator. A modified Luggin capillary separated the working compartment of the electrochemical cell from the reference compartment. All experiments were carried out under an inert atmosphere by bubbling argon through the working compartment.

## RESULTS AND DISCUSSION

In order to obtain mechanistic information concerning the electrocatalytic reduction of ferricyanide at DNA-modified electrodes, electrochemical measurements were carried out using rotated disk voltammetry (8). At a rotated disk electrode (RDE), the mass transfer limited current ( $i_L$ ) depends only on the angular rotational velocity ( $\omega$ ) and the bulk concentration of ferricyanide in solution,  $[\text{Fe}(\text{CN})_6^{3-}]$  ( $\text{mol}/\text{cm}^3$ ), according to the Levich equation (9):

$$i_L = 0.62nFAD^{2/3}[\text{Fe}(\text{CN})_6^{3-}]^* \omega^{1/6} \nu^{1/2}$$

Here  $n$  is the number of electrons transferred,  $F$  is Faraday's constant,  $A$  is the electrode area,  $\eta$  is the kinematic viscosity, and  $D$  is the diffusion constant.

For all calculations, the angular velocity is taken as  $\omega = (2\pi/60) \cdot f$ , where  $f$  is the frequency of rotation in rpm, and values for  $\omega^{1/6}$  and  $D$  are 2.154 and  $7.9 \times 10^{-6} \text{ cm}^2/\text{s}$ , respectively). A linear plot of  $i_L$  vs.  $\omega^{1/2}$  (a Levich Plot) implies that the electrocatalytic reaction is faster than the rate of substrate delivery to the electrode, so that the current is determined only by how fast the substrate is transported to the surface.

Figure 5.1 shows the RDE linear sweep voltammetry of 0.3 mM  $\text{Fe}(\text{CN})_6^{3-}$  at a DNA-modified electrode (TA sequence) in the presence of 4 and 8  $\mu\text{M}$   $\text{MB}^+$ . In each case, the current begins at zero, then rises sharply at the formal reduction potential of methylene blue before peaking and finally diminishing to a stable plateau value. Reversing the scan direction reveals more typical RDE  $i$ - $V$  traces, as this "peak" is no longer present. At higher rotation rates, the effective concentration of ferricyanide at the electrode surface increases, so the limiting current rises as well. Interestingly, both higher rotation rates and lower concentrations of  $\text{MB}^+$  in solution lead to greater deviations from the idealized response, resulting in more prominent peaks and greater attenuations from the transport controlled steady state currents. This is illustrated in the Levich Plots shown in Figure 5.2; under strictly diffusion controlled conditions, these plots would be linear and pass through the origin. Here, however, the electrocatalysis cannot keep up with the increased flux of  $\text{Fe}(\text{CN})_6^{3-}$  at high rotation rates, so the currents level off.

For a reaction mediated by a surface-bound cofactor (in this case, MB<sup>+</sup>), the kinetically controlled current ( $i_{kin}$ ) is given by (9):

$$i_{kin} = nFA[\text{Fe}(\text{CN})_6^{3-}]\Gamma_{\text{MB}}k_{\text{obs}} \quad 2$$

where  $\Gamma_{\text{MB}}$  is the surface coverage of the mediator (MB<sup>+</sup>) bound to the electrode surface, and  $k_{\text{obs}}$  is the pseudo second order rate constant. So combining equations 1 and 2 leads to the Koutecky-Levich expression (9), which describes the overall RDE plateau current for the electrocatalytic reaction:

$$\begin{aligned} i_{lim}^{-1} &= i_L^{-1} + i_{kin}^{-1} \\ &= (0.62nFAD^{2/3}[\text{Fe}(\text{CN})_6^{3-}]\omega^{1/6}w^{1/2})^{-1} + (nFA[\text{Fe}(\text{CN})_6^{3-}]\Gamma_{\text{MB}}k_{\text{obs}})^{-1} \end{aligned} \quad 3$$

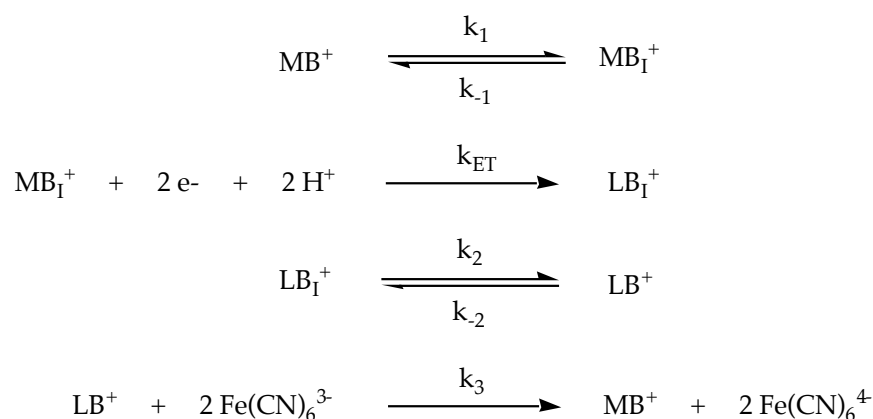
According to this expression, a plot of  $i_{lim}$  vs.  $\omega^{1/2}$  should yield a straight line whose slope is related to the number of electrons transferred ( $n$ ) and whose intercept gives the product  $\Gamma \cdot k_{\text{obs}}$ . Because the electrochemistry of MB<sup>+</sup> at DNA-modified surfaces with no  $\text{Fe}(\text{CN})_6^{3-}$  present provides an independent measure of  $\Gamma_{\text{MB}}$ , RDE voltammetry provides a convenient method for evaluating  $k_{\text{obs}}$  directly.

Figure 5.3 shows Koutecky-Levich plots for the electrocatalytic reduction of  $\text{Fe}(\text{CN})_6^{3-}$  as a function of [MB<sup>+</sup>] in solution. Each concentration yields a straight line parallel to the theoretical curve drawn for  $n = 1$ , but with decreasing intercepts as the total concentration of MB<sup>+</sup> increases. Indeed, at

[MB<sup>+</sup>] greater than  $\sim 15 \mu\text{M}$ , the Levich plots become linear and the Koutecky-Levich plots pass through the origin, implying a purely mass transport controlled reaction. Using  $\Gamma_{\text{MB}} = 50(5) \text{ pmol/cm}^2$ , as determined by CV, we calculate second order rate constants,  $k_{\text{obs}}$ , ranging from  $3.2 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$  ( $2 \mu\text{M}$  MB<sup>+</sup>) to  $4.3 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$  ( $12 \mu\text{M}$  MB<sup>+</sup>). These data are summarized in Table 5.1.

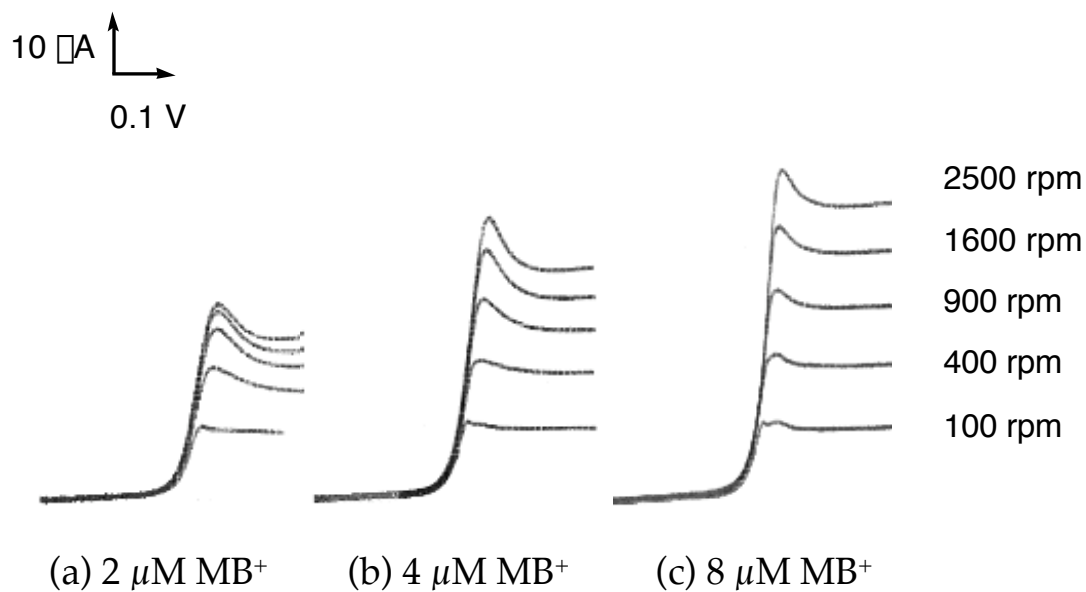
Consistent with the *i*-V traces shown in Figure 5.1, these plots demonstrate that the overall catalytic rate is strongly dependent on the concentration of MB<sup>+</sup>. A plot of  $k_{\text{obs}}$  vs. [MB<sup>+</sup>] is shown in Figure 5.4, revealing a first order dependence in total [MB<sup>+</sup>]. Qualitatively, these data suggest that the DNA film is initially loaded with a relatively high concentration of MB<sup>+</sup> catalyst before the linear sweep. Thus as the potential is swept past the formal  $E^\circ$  of MB<sup>+</sup>, there is a catalytic burst in the turnover of  $\text{Fe}(\text{CN})_6^{3-}$ , which depletes the surface concentration of catalyst causing a peak in the RDE voltammogram. Once depleted, the reaction is limited by how fast the active form of the catalyst can be regenerated, resulting in a final steady state catalytic current which is less than predicted by equation 1.

Given this scenario, we propose the following general mechanism for the overall electrocatalytic response:

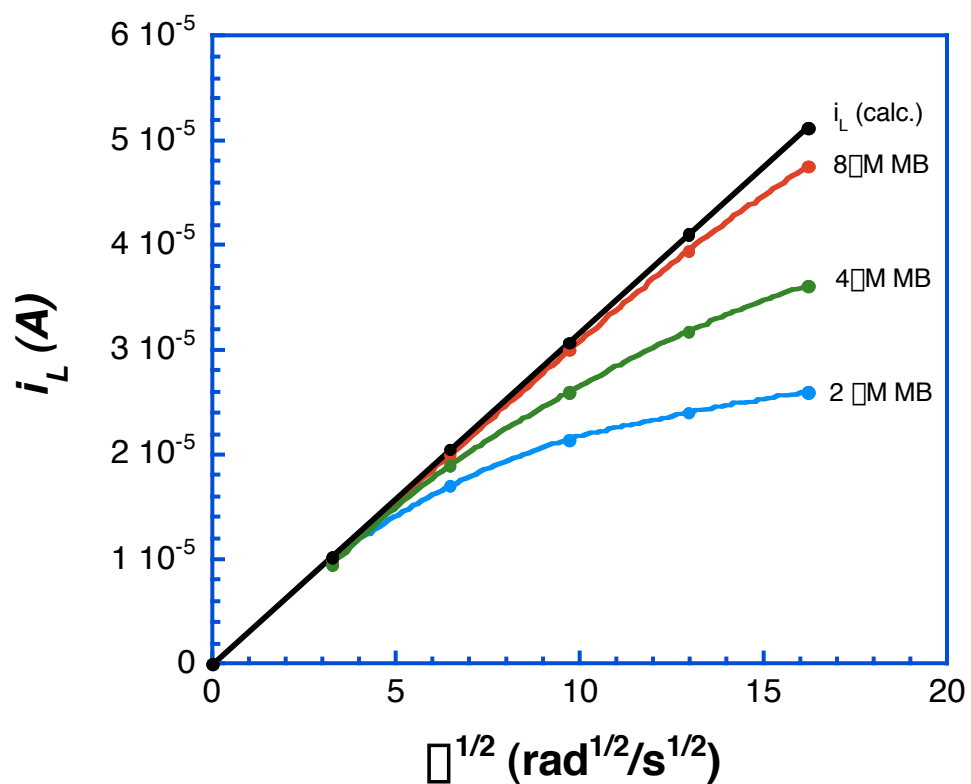




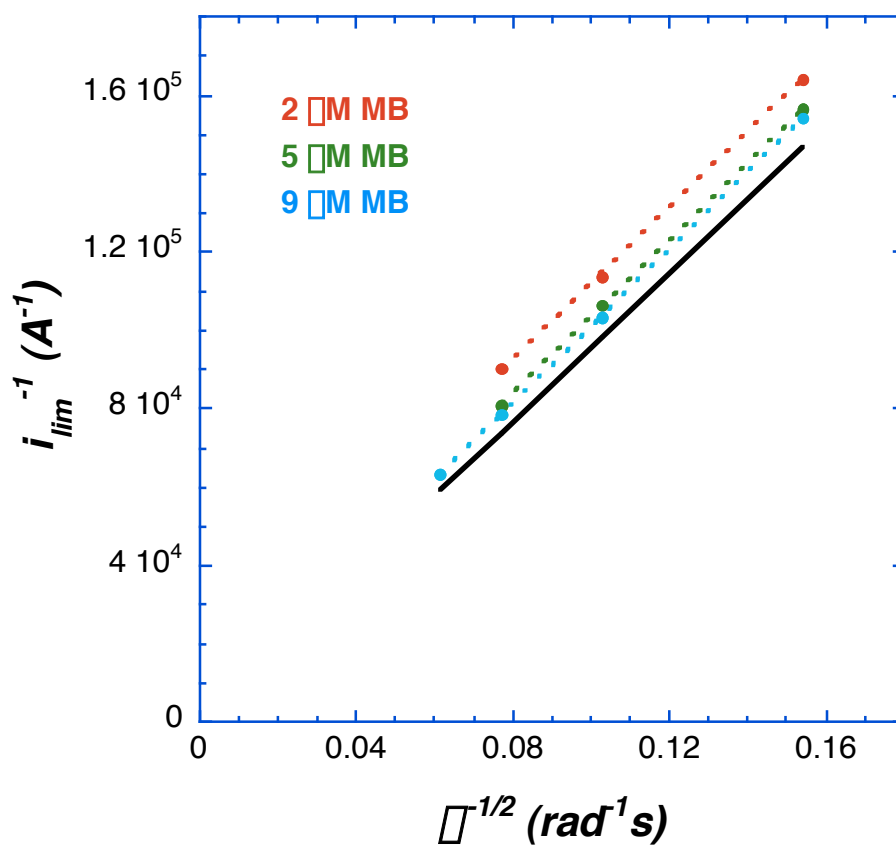
**Figure 5.1.** Linear sweep RDE voltammetry of 0.3 mM  $\text{Fe}(\text{CN})_6^{3-}$  in the presence of (a) 2  $\mu\text{M}$   $\text{MB}^+$ , (b) 4  $\mu\text{M}$   $\text{MB}^+$ , and (c) 8  $\mu\text{M}$   $\text{MB}^+$  at a DNA-modified electrode (TA sequence). Rotation rates = 100, 400, 900, 1600, and 2500 rpm;  $\nu = 2$  mV/s.



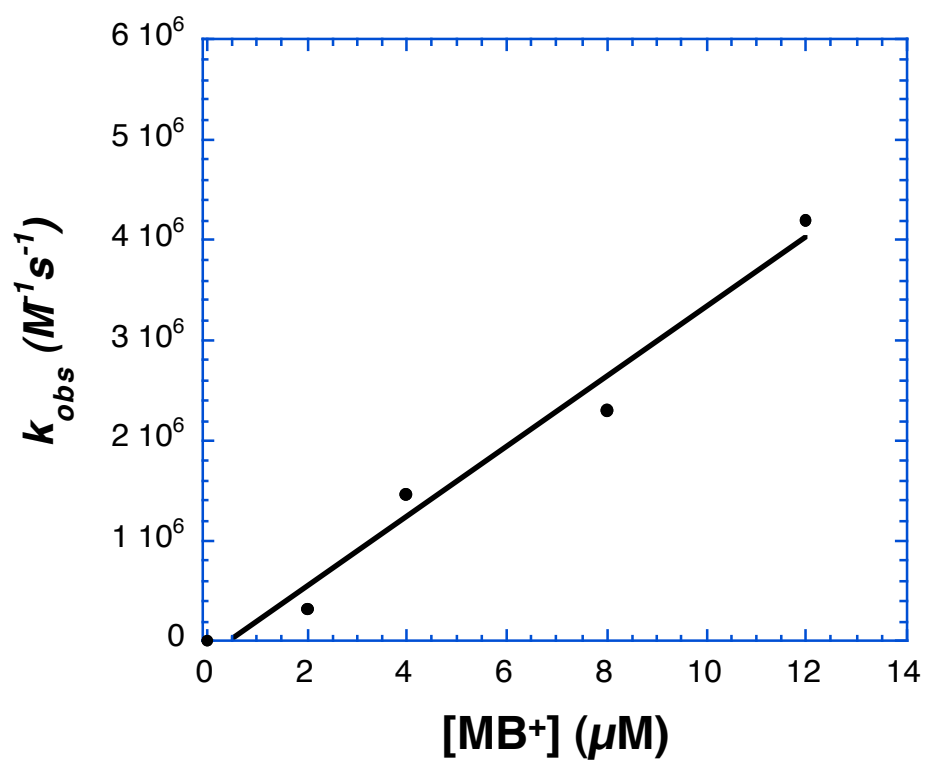
**Figure 5.2.** Levich plots for the electrocatalytic reduction of  $\text{Fe}(\text{CN})_6^{3-}$  at a DNA-modified electrode (TA sequence) in the presence of 2, 4, and 8  $\mu\text{M}$   $\text{MB}^+$ . Plateau currents were measured at  $-400$  mV. The idealized response for a diffusion controlled process is shown in black.



**Figure 5.3.** Koutecky-Levich plots for the reduction of  $\text{Fe}(\text{CN})_6^{3-}$  at a DNA-modified electrode (TA sequence) in the presence of 2, 5, and 9  $\mu\text{M}$   $\text{MB}^+$ . The theoretical curve for a diffusion controlled response is shown in black.



**Figure 5.4.** Plot of  $k_{\text{obs}}$  vs.  $[\text{MB}^+]$  for  $\text{MB}^+$ -mediated reduction of  $\text{Fe}(\text{CN})_6^{3-}$  at a DNA-modified electrode (TA sequence). Values for  $k_{\text{obs}}$  were calculated using a  $\Gamma_{\text{MB}}$  of 52 pmol/cm<sup>2</sup> as determined by cyclic voltammetry.



In this mechanism,  $\text{MB}^+$  free in solution first intercalates into the surface bound DNA duplexes ( $\text{MB}^+_{\text{I}}$ ) to become electrochemically active ( $k_1$ ). When the potential is poised at sufficiently negative values,  $\text{MB}^+_{\text{I}}$  undergoes a two electron reduction ( $k_{\text{ET}}$ ) to leucomethylene blue ( $\text{LB}^+_{\text{I}}$ ). Some of the  $\text{LB}^+_{\text{I}}$  then dissociates from the film ( $k_2$ ) where it is immediately oxidized by two equivalents of  $\text{Fe}(\text{CN})_6^{3-}$  to regenerate  $\text{MB}^+$  and complete the catalytic cycle.

An interesting feature of this mechanism is that the overall reaction relies on catalyst (rather than substrate) binding and release from the surface. This notion is fully consistent with several key experimental observations. For example, our previous work has shown that  $\text{MB}^+$  binds reversibly to DNA-modified surfaces (obeying a Langmuir Isotherm), with an affinity nearly identical to DNA in solution, but upon reduction the affinity is significantly less so some of it dissociates from the film (1). Moreover, we have found that redox probes that intercalate too deeply into DNA, or exhibit sluggish dissociation kinetics (e.g., daunomycin) are not effective mediators for the electrocatalysis (Chapter 2). These findings support the idea that  $\text{LB}^+_{\text{I}}$  must at least partially dissociate from the film so that it can come into contact with the ferricyanide in solution to affect the electrocatalytic reduction.

Assuming that  $\text{LB}^+$  (reduced  $\text{MB}^+$ ) is the active form of the catalyst that reduces  $\text{Fe}(\text{CN})_6^{3-}$ , the overall reaction rate is given by equation 4:

$$\square_{\text{prod}} = 2k_3[\text{Fe}(\text{CN})_6^{3-}][\text{LB}^+] \quad 4$$

Using the steady state approximation, 4 becomes 5:

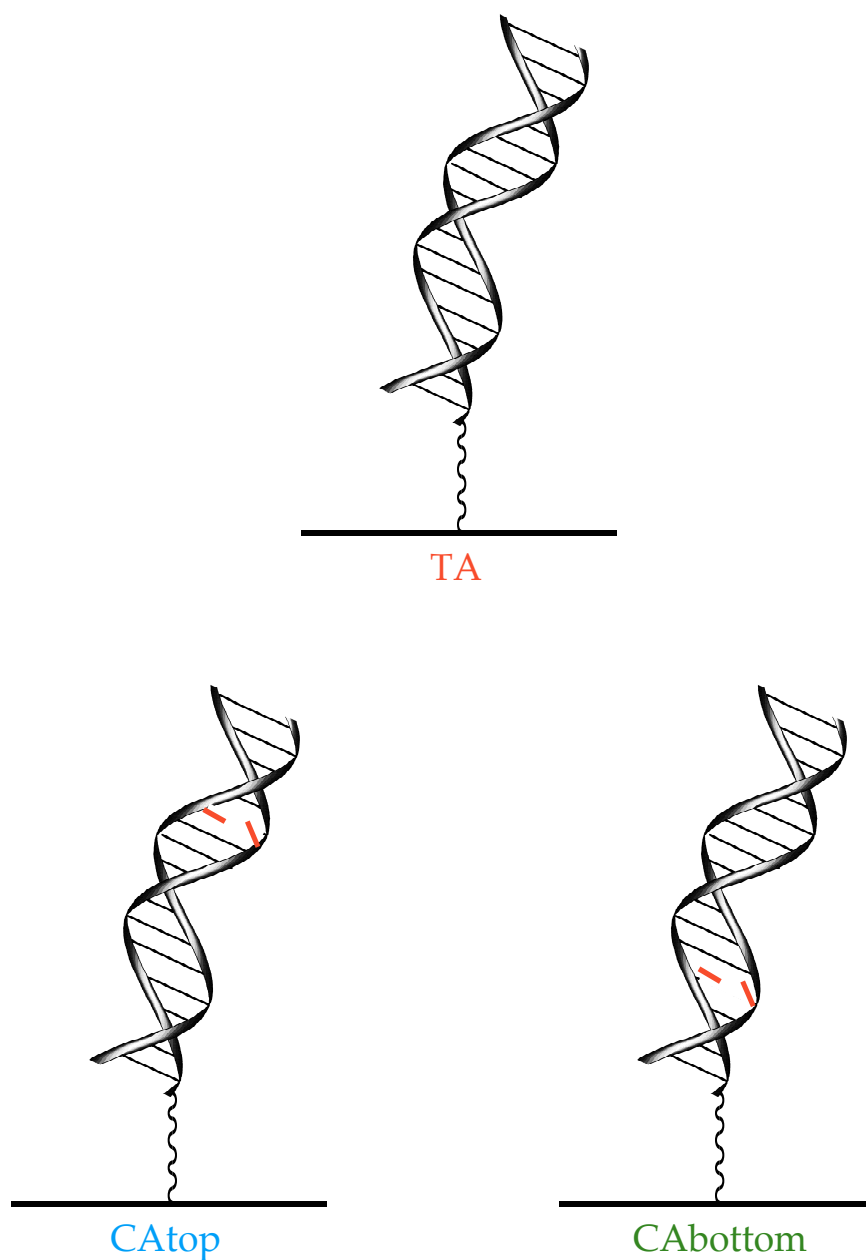
$$j_{prod} = \frac{2k_3[Fe(CN)_6^{3-}][MB]_{total}}{2k_3[Fe(CN)_6^{3-}]\left(\frac{1}{k_1} + \frac{k_{01}}{k_1}\frac{1}{k_{ET}}\right) + \frac{1}{k_{ET}} + \frac{1}{k_2}\frac{1}{k_{02}} + 1 + \frac{k_{02}}{k_2}}$$
5

According to this expression, the rate should be first order in total MB<sup>+</sup> concentration (as we experimentally observed, Figure 5.4), and is ultimately governed by the sum  $k_1 + k_2$ , clearly indicating that  $k_{ET}$ , the rate of charge transport through the DNA, is not the rate limiting step in this system. Whether diffusion of MB<sup>+</sup> into the film ( $k_1$ ) or dissociation of LB<sub>1</sub><sup>+</sup> out of the film ( $k_2$ ) is the actual rate determining step cannot be resolved from this rate law. However, regardless of the relative magnitudes of  $k_1$  and  $k_2$ , equation 5 leads to several important predictions. For example, it has been shown previously that the electrocatalytic currents are greatly diminished if the individual DNA duplexes within the electrode films possess a base mismatch (Chapters 2 and 3, references 6 and 7). To account for this behavior, we have qualitatively proposed that the methylene blue must intercalate *below* the location of the mismatch to be electrochemically active. Thus the deeper the mismatch, the further into the film the MB<sup>+</sup> must diffuse, and the slower the overall reaction.

To examine this quantitatively, we have prepared several DNA films that feature CA mismatches at various locations along the helix (Figure 5.5). If our model is correct, the electrocatalytic rates should decrease along the series TA > CAtop > CAbottom, owing to slower diffusion rates of MB<sup>+</sup> and LB<sub>1</sub><sup>+</sup> into and out of the films ( $k_1$  and  $k_2$ ).

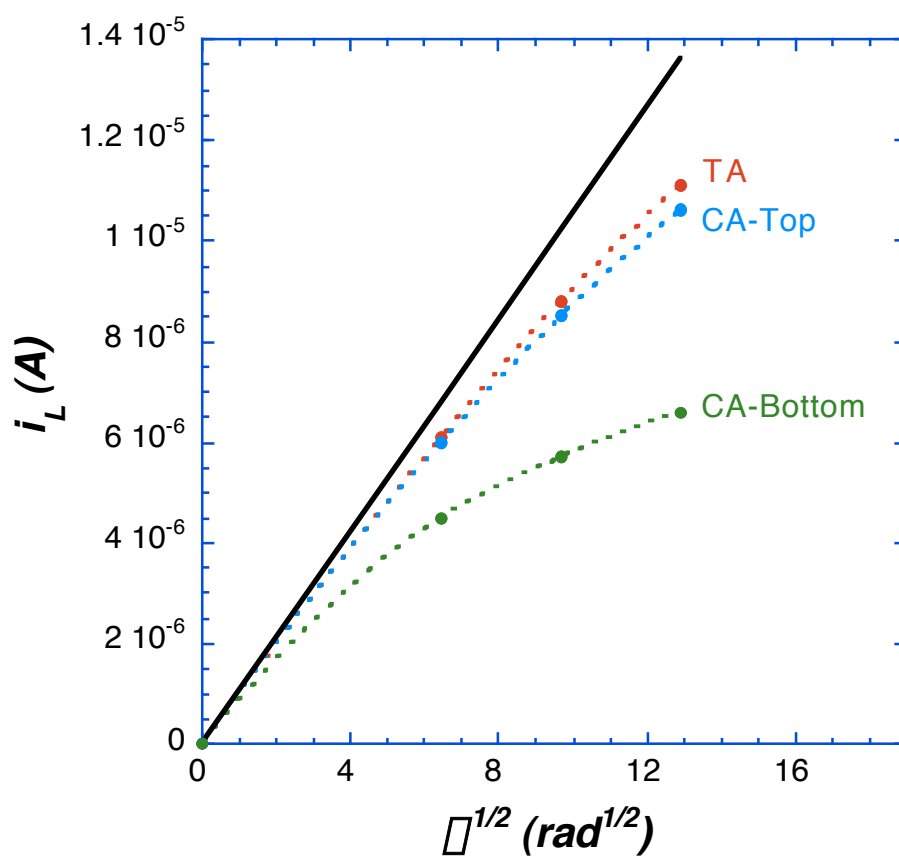
Indeed, this is precisely what we find. Figure 5.6 shows the Levich plots for the RDE voltammetry of  $0.3 \text{ mM Fe(CN)}_6^{3-}$  in the presence of  $2 \text{ }\mu\text{M MB}^+$  at electrodes comprised of the TA sequence, CAtop, and CAbottom. The catalysis shows the same type of dependence on  $[\text{MB}^+]_{\text{total}}$  at each electrode, but the overall currents (and therefore the overall catalytic rates) decrease as the mismatch is moved further down the helix. Koutecky-Levich plots (Figure 5.7) for these systems confirm this and yield the data summarized in Table 5.1. Figure 5.8 shows a plot of  $k_{\text{obs}}$  vs.  $[\text{MB}^+]$  for TA, CAtop, and CAbottom. These data clearly show that the intercalator diffusion into and/or out of the film depends on the location of the mismatch. The rate is slower, the deeper into the film the mismatch is located, and thus the farther into and out of the film  $\text{MB}^+$  must diffuse to be reduced and participate in catalysis.

**Figure 5.5.** Schematic representation of surface-bound DNA duplexes featuring mismatches at different locations within the helix. The thiol modified sequence used is 5'-SH-AGTACAGTTCATCGCG-3'. When hybridized to its perfect complement, the duplex is referred to as TA, hybridized to a complement with a CA mismatch at the boldface C is referred to as CAtop, and hybridized to a complement with a CA mismatch at the underlined C is referred to as CAbottom.

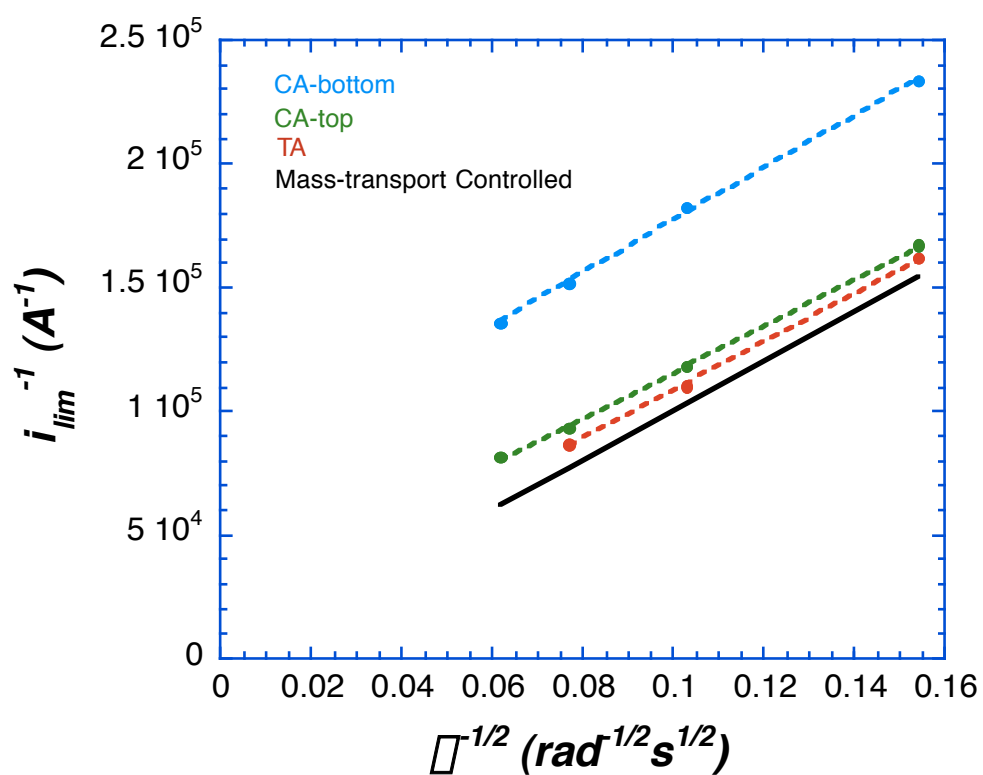




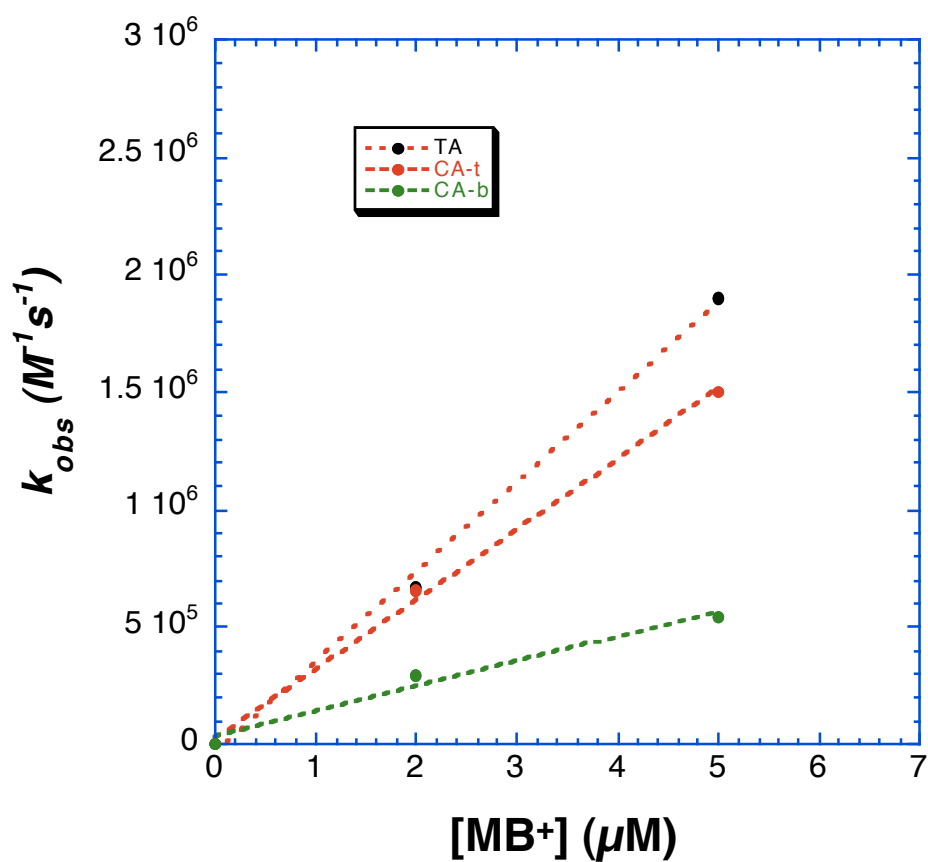
**Figure 5.6.** Levich plots for the electrocatalytic reduction of  $\text{Fe}(\text{CN})_6^{3-}$  at a DNA-modified electrode with TA, CA<sub>top</sub>, and CA<sub>bottom</sub> sequences in the presence of  $2 \mu\text{M}$  MB<sup>+</sup>. Plateau currents were measured at  $-400$  mV. The idealized response for a diffusion controlled process is shown in black.



**Figure 5.7.** Koutecky-Levich plots for the reduction of  $\text{Fe}(\text{CN})_6^{3-}$  at a DNA-modified electrode with TA, CA-top, and CABottom sequences in the presence of  $8 \mu\text{M MB}^+$ . The theoretical curve for a diffusion controlled response is shown in black.



**Figure 5.8.** Plot of  $k_{\text{obs}}$  vs.  $[\text{MB}^+]$  for  $\text{MB}^+$ -mediated reduction of  $\text{Fe}(\text{CN})_6^{3-}$  at a DNA-modified electrode with TA, CA<sub>top</sub>, and CA<sub>bottom</sub> sequences. Values for  $k_{\text{obs}}$  were calculated using a  $\Gamma_{\text{MB}}$  of 52 pmol/cm<sup>2</sup> as determined by cyclic voltammetry.



**Table 5.1.** Rate data for electrocatalysis of  $\text{Fe}(\text{CN})_6^{3-}$  at DNA-modified electrodes.

Sequence	$[\text{MB}^+]$ ( $\mu\text{M}$ )	$\Gamma_{\text{MB}}^{\text{a}}$ ( $\text{pmol}/\text{cm}^2$ )	$k_{\text{obs}}^{\text{b}}$ ( $\text{M}^{-1}\text{s}^{-1}$ )
TA	2	50 (5)	$6.7(7) \times 10^5$
	5		$1.9(2) \times 10^6$
	9		$4.7(5) \times 10^6$
CA <sub>top</sub>	2	42 (7)	$6.5(9) \times 10^5$
	5		$1.5(2) \times 10^6$
	9		$4.6(7) \times 10^6$
CA <sub>bottom</sub>	2	22 (8)	$2.9(7) \times 10^5$
	5		$5.4(9) \times 10^5$
	8		$1.0(10) \times 10^6$

<sup>a</sup> Determined by cyclic voltammetry in the absence of  $\text{Fe}(\text{CN})_6^{3-}$ .

<sup>b</sup> Measured at a RDE using 0.1 mM  $\text{Fe}(\text{CN})_6^{3-}$ .

## SUMMARY

We have developed a sensitive method for the detection of single base mismatches based on the electrocatalytic reduction of  $\text{Fe}(\text{CN})_6^{3-}$  by  $\text{MB}^+$  bound to DNA-modified electrodes. In order to obtain mechanistic information about this electrocatalytic reaction, electrochemical measurements were carried out using rotated disk voltammetry. Using Koutechy-Levich analyses of data obtained with linear sweep RDE voltammetry at various  $[\text{MB}^+]$  and rotation rates, observed rate constants were measured for perfectly matched as well as mismatched DNA films. An overall rate law for this electrocatalytic reaction was derived. According to this expression, the rate is governed by the total concentration of  $\text{MB}^+$  as well as the sum of the rates of  $\text{MB}^+$  binding to DNA and reduced  $\text{MB}^+$  disassociating from the DNA film. This model is fully consistent with all of our experimental observations in this  $\text{MB}^+/\text{Fe}(\text{CN})_6^{3-}$  electrocatalytic system and underscores the enhanced sensitivity to mismatches seen with electrocatalytic cycling versus direct electrochemistry of DNA intercalators (Chapters 2 and 3). Furthermore, this mechanism may have implications for electrocatalysis in other DNA electrochemistry systems (10).

## REFERENCES

1. Kelley, S.O., Barton, J.K., Jackson, N.M., Hill, M.G. (1997) *Bioconj. Chem.* 8, 31-37.
2. Kelley, S.O., Barton, J.K., Jackson, N.M., McPherson, L.D., Potter, A.B., Spain, E.M., Allen, M.J., Hill, M.G. (1998) *Langmuir* 14, 6781-6784.
3. Boon, E.M., Barton, J.K., Sam, M., Hill, M.G., Spain, E.M. (2001) *Langmuir* 17, 5727-5730.
4. Kelley, S.O., Jackson, N.M., Hill, M.G., Barton, J.K. (1999) *Angew. Chem. Int. Ed.* 38, 941-945.
5. Kelley, S.O., Boon, E.M., Barton, J.K., Jackson, N.M., Hill, M.G. (1999) *Nuc. Acids Res.* 27, 4830-4837.
6. Boon, E.M., Ceres, D.M., Drummond, T.G., Hill, M.G., Barton, J.K. (2000) *Nature Biotech.* 18, 1096-1100.
7. Boon, E.M., Salas, J.E., Barton, J.K. (2001) *Nature Biotech.* 20, 282-286.
8. Steiger, B., Shi, C., Anson, F.C. (1993) *Inorganic Chemistry* 32, 2107-2113.
9. Bard, A.J., Faulkner, L.R. (1980) "Electrochemical Methods." Wiley, New York.
10. Ropp, P.A., Thorp, H.H. (1999) *Chem. Biol.* 6, 599-605.